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Differences that Matter: Major Cytotoxic T Cell–Stimulating Minor Histocompatibility Antigens

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Summary

Despite thousands of genetic polymorphisms among MHC matched mouse strains, a few unknown histocompatibility antigens are targeted by the cytotoxic T cells specific for tissue grafts. We isolated the cDNA of a novel BALB.B antigen gene that defines the polymorphic *H28* locus on chromosome 3 and yields the naturally processed ILENFPRL (IFL8) peptide for presentation by K^b MHC to C57Bl/6 CTL. The CTL specific for the IFL8/K^b and our previously identified H60/K^b complexes represent a major fraction of the B6 anti-BALB.B immune response. The immunodominance of these antigens can be explained by their differential transcription in the donor versus the host strains and their expression in professional donor antigen-presenting cells.

Introduction

Even a few polymorphic amino acid substitutions in the MHC molecules impose an insurmountable barrier to the acceptance of tissue grafts by normal individuals (Snell, 1948). However, when the MHC loci are identical between the donor and the host, tissue grafts can still be rejected due to equally imposing barriers posed by other polymorphic differences–the minor histocompatibility loci (*H*). Due to their central importance in determining the final outcome of clinical transplants, the *H* loci have been intensively studied for decades (Little, 1914). At least 60 *H* loci have been genetically identified in mice and have been mapped to the mitochondrial as well as the sex and autosomal chromosomes (Loveland et al., 1990; Simpson et al., 1997). The *H* loci are the source of antigenic peptides presented by the classical as well as the nonclassical MHC molecules in cells of the tissue donor or the recipient of a bone marrow graft (Loveland et al., 1990; Wallny and Rammensee, 1990). The resulting T cell responses to these key H peptide/MHC complexes subsequently cause chronic graft rejection or graft versus host disease. Identifying the antigen peptides and their genetic sources is therefore central to understanding H antigens at a molecular level and to elucidating the mechanisms underlying their immunogenicity.

Non-MHC polymorphisms that cause histoincompatibility have been most extensively studied in the C57BL/6 and BALB.B strains. These strains are identical in their MHC but differ extensively in other background genes. Estimates of the number of H antigen polymorphisms that distinguish these two strains have extended into the hundreds (Bailey and Mobraaten, 1969; Bailey, 1999). However; the B6 anti-BALB.B $CD8^+$ cytotoxic T cell (CTL) responses appear to be directed against a small subset of the total potential H antigens (Wolpert et al., 1995; Nevala and Wettstein, 1996). Until recently, none of these antigens were known at the molecular level, thereby making it impossible to understand their unique characteristics that confer immunogenicity and immunodominance. Using an expression cloning strategy, we recently isolated a cDNA clone that defined the *H60* locus and identified its naturally processed peptide LYL8/K^b MHC complex as the ligand for B6 anti-BALB.B CTL (Malarkannan et al., 1998). Here, we used this expression cloning approach to isolate a cDNA that defines a distinct *H* locus, *H28*, and encodes a previously unknown protein that yields the naturally processed ILENFPRL (IFL8) octapeptide presented by the K^b MHC molecule. We provide direct evidence that the H60 and H28 antigens dominate the CD8 T response in B6 mice immunized with BALB.B spleen cells. Unlike all the other known *H* loci, the *H28* and *H60* genes are transcribed in the BALB.B donor but not in host B6 cells. Both antigens are expressed in professional APCs in the donor strain; H60 constitutively and H28 after induction with inflammatory cytokines. The findings provide the basis for the emerging paradigms for immunity to histocompatibility antigens.

Results

Isolation of B6 Anti-BALB.B $CD8^+$ T Cell Clone and Its Target Antigen Gene

To probe the polymorphic antigenic differences between the C57Bl/6 (B6) and its MHC matched BALB.B strains, we first established a polyclonal cytotoxic T cell line from the spleens of B6 mice immunized with BALB.B spleen cells. The CTL line was fused to BWZ.36/CD8α to generate lacZ inducible T cell hybrids (Sanderson and Shastri, 1994). One of these hybridomas, BCZ104.2, was selected because it specifically responded to the donor BALB.B spleen cells (Figure 1A) but failed to recognize our previously identified H60 locus derived peptide (Malarkannan et al., 1998). The response of the BCZ104.2 T cells required the K^b MHC molecule, because it was inhibited by an anti-K^b monoclonal antibody but not with anti-D^b or with anti-A^b antibodies (Figure 1B). The BCZ104.2 T cell was therefore specific for a polymorphic antigen presented by the K^b MHC I molecule in the donor BALB.B spleen cells.

To identify its cognate antigen, the BCZ104.2 T cell hybrid was used to probe a cDNA expression library prepared from cells derived from (BALB/c × BALB.B)F1 mice. The library was screened in pools of ~50 cDNAs/well together with the K^b gene in recipient COS cells.

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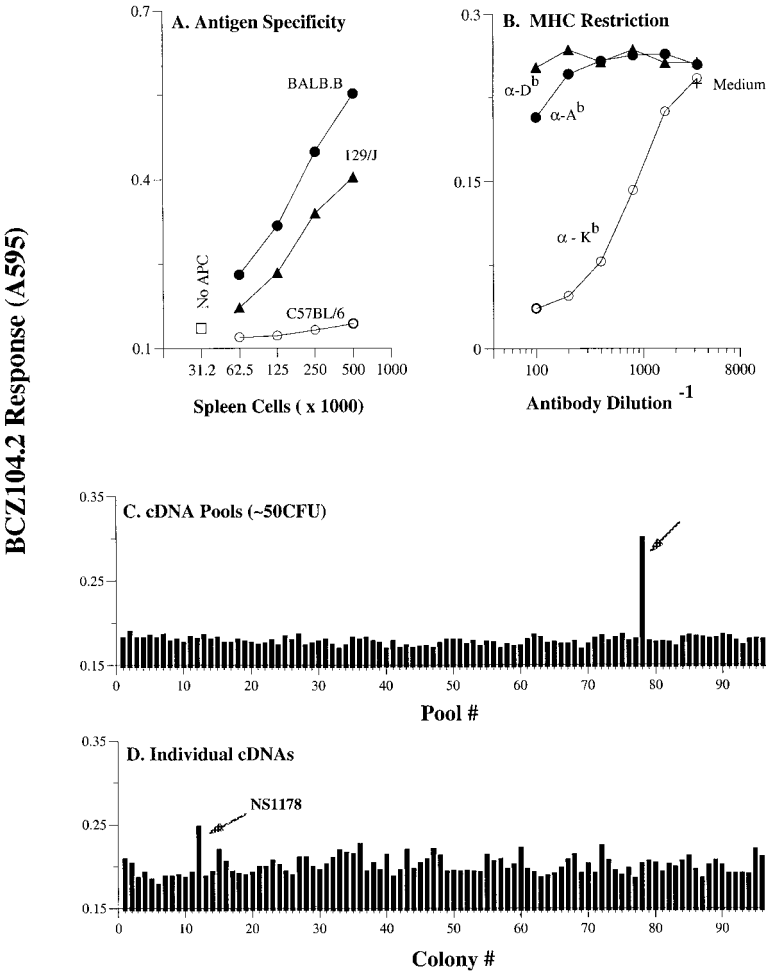

Figure 1. Generation of B6 Anti-BALB.B T Cell Hybrid and Isolation of Its Antigen Gene

The BCZ104.2 T cell hybrid recognizes a polymorphic H antigen expressed in BALB and 129/J backgrounds that is presented by the K^b MHC molecule. (A) 1×10^5 BCZ104.2 T cells were cocultured overnight with varying numbers of spleen cells from the indicated strains or (B) with 6×10^4 BALB.B cells and dilutions of anti-K^b, D^b, or A^b monoclonal antibodies. The T cell response was measured by the induction of lacZ activity by the conversion of the substrate chlorophenolred-β-D-galactopyranoside (CPRG) at 595 nm and 655 nm wavelength as reference. (C) Plasmids from a (BALB/cXBALB.B)F1 cDNA library were transiently transfected into 3×10^4 COS cells as pools or (D) from individual colonies of the positive pool together with K^b cDNA. Two days later, BCZ104.2 T cells were added, and after overnight culture their lacZ response was measured as above.

Two days later, the BCZ104.2 T cells were added and their response measured by the induced β-galactosidase activity. Among a total of ~10^5 cDNA pools tested, the response of BCZ104.2 T cells to one of the pools was clearly above background (Figure 1C). Individual bacterial colonies, obtained by retransforming the DNA mixture from this pool were rescreened, and the plasmid NS1178 was identified as capable of stimulating the BCZ104.2 T cells (Figure 1D). The BCZ104.2 T cell response to COS cells transfected with the NS1178 cDNA was specific and required coexpression of the K^b MHC I molecule (see below). We conclude that the NS1178 cDNA was a likely candidate encoding the BALB antigen that was recognized by the K^b restricted BCZ104.2 T cell.

NS1178 cDNA Encodes a Novel Gene Product and Maps the Antigen Gene to the Differentially Transcribed Polymorphic *H28* Locus

The nucleotide sequence of the NS1178 cDNA clone was determined to characterize the donor gene and its putative protein product. The 2279 nucleotide sequence of NS1178 revealed a 1320 nucleotide open reading frame encoding 449 amino acids (Figure 2A). To characterize the putative protein product the NS1178 cDNA was used as a template in an in vitro transcription/translation assay. Analysis of the labeled products revealed a band of ~50 kDa showing that the first ATG codon was predominantly used for translation initiation (Figure 2B). Likewise, the previously isolated H60 cDNA yielded a band corresponding to ~30 kDa, confirming that this cDNA also yields a protein with the expected molecular weight (Malarkannan et al., 1998).

Comparison of the NS1178 protein sequence with those in the sequence databases did not yield an exact match to any known gene (http://www.ncbi.nlm.nih.gov). The closest homology with 49% amino acid identities was to a hypothetical protein (NP006811.1) expressed in human osteoblasts and with 36% amino acid identities to four other proteins (P27473, A43676, NP006408.1, S48218). The latter proteins have been characterized as microtubular aggregate proteins associated with hepatitis antigens in humans and nonhuman primates. These similarities and the absence of membrane translocation or nuclear localization signals suggest that NS1178 encodes a cytoplasmic protein in contrast to H60, which, based upon its sequence, is a type I transmembrane protein.

To define the molecular basis of the antigenic polymorphism at the NS1178 locus, we examined restriction fragment length polymorphisms (RFLP). Southern blots of genomic DNA from the BALB.B and B6 strains probed with the NS1178 cDNA revealed HindIII, BamHI, and XbaI RFLP (data not shown). The strain distribution pattern of

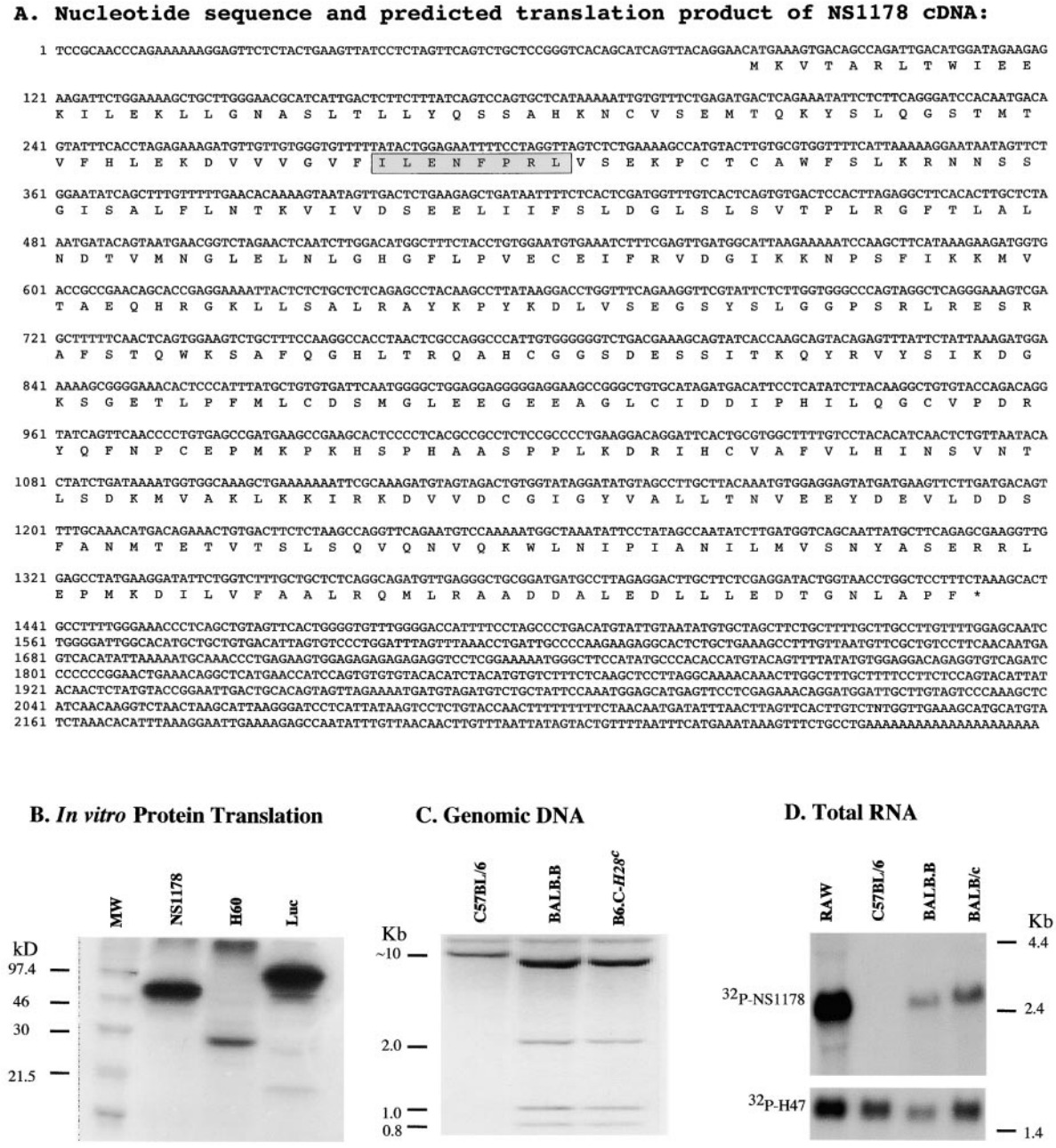

Figure 2. NS1178 cDNA Encodes a Novel Protein that Defines the Polymorphic, Differentially Transcribed *H28* Locus

(A) Nucleotide sequence and predicted translation product of the NS1178 cDNA. The antigenic peptide is boxed.

(B) Products of in vitro protein translation reactions using the NS1178, *H60*, and control luciferase cDNAs.

(C) Southern blot analysis of genomic DNA from C57Bl/6, BALB.B, or the congenic B6.C-*H28*^{c} digested with XbaI and probed with ^{32}P-labeled NS1178 probe. Molecular weight markers are indicated on the left.

(D) Northern blot analysis of total RNA from the BALB.B, BALB/c, C57Bl/6 spleen cells, or IFNγ-induced RAW cells probed with ^{32}P-labeled NS1178 or H47 cDNA probes. Molecular weight markers are indicated on the right.

DNAs from a panel of BxC recombinant inbred lines derived from the parental BALB/c and B6 strains suggested a linkage concordant with an interval on chromosome 3 containing the *H28* locus. This linkage was consistent with a pattern previously described for the CTT-3 and IDE-3 antigens by Karre and Wettstein laboratories (Wolpert et al., 1995; Nevala and Wettstein, 1996). To test this hypothesis directly, we examined RFLP in DNA from the B6, BALB.B, and the B6.C-*H28*^{c} congenic strain (Bailey, 1975). In contrast to the single ~10 kb band in the B6 strain, each of the four (8, 2, 1, and 0.8 kb) bands detected in the BALB.B DNA were also found in the B10.C-*H28*^{c} strain (Figure 2C). We conclude that the NS1178 cDNA detected a polymorphic locus that segregated with the *H28* congenic interval.

Northern blot analysis with the NS1178 probe in

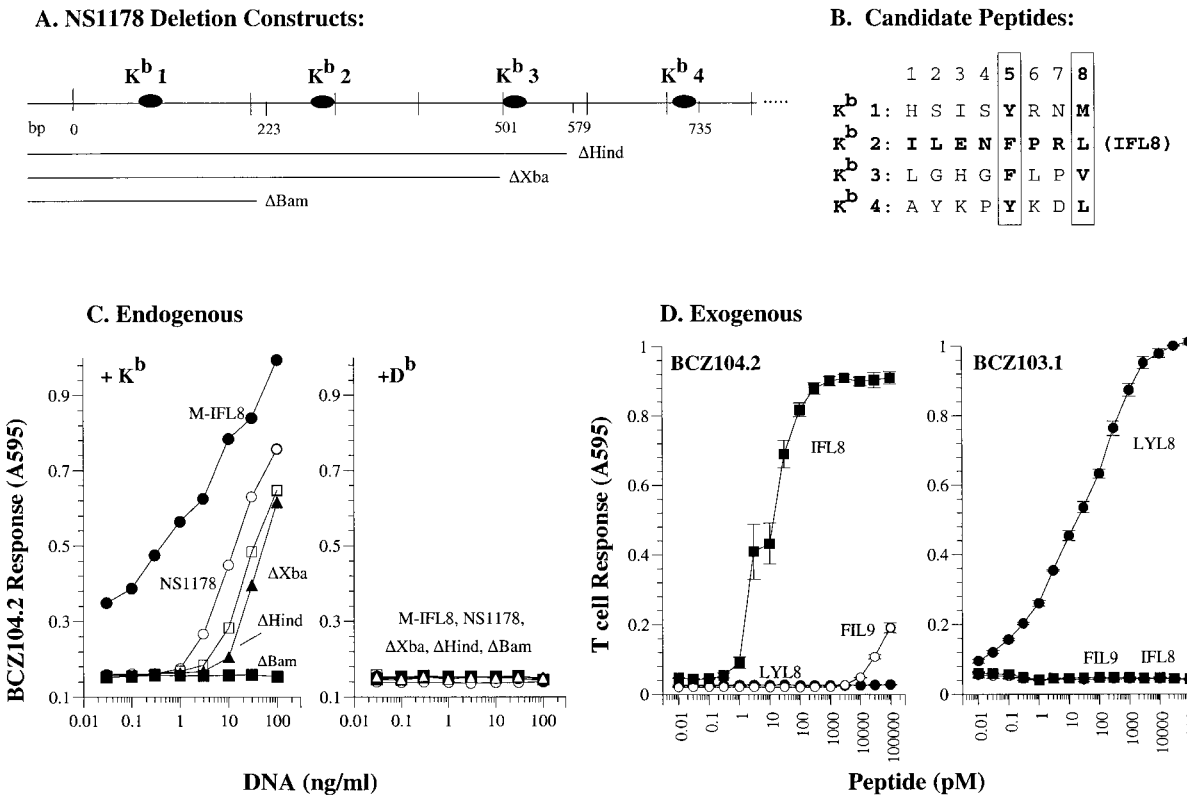

Figure 3. The IFL8 Octapeptide Is the Minimal Antigenic Peptide within the NS1178 Gene Product

(A) Schematic representation of deletion constructs of the NS1178 cDNA.

(B) Sequences of candidate antigenic peptides as predicted by the K^b binding motif.

(C) Plasmid DNAs of the indicated deletion constructs or the minigenes encoding IFL8 antigenic peptide were transiently transfected into COS cells together with either K^b MHC or D^b MHC cDNA. The BCZ104.2 T cells were added after 48 hr and assayed for lacZ activity the next day.

(D) The lacZ response of either BCZ104.2 or the H60-specific BCZ103.1 T cells after overnight culture with K^b-L cells and varying concentrations of either synthetic IFL8 (ILENFPRL), FFL9 (F-IFL8) peptides, or the H60 derived peptide LYL8 (LTFNYRNL). The lacZ responses were measured as in legend to Figure 1.

spleen cells showed that this gene was transcribed in the spleen cells and in interferon-γ-induced RAW cells derived from the BALB background. In contrast, transcripts for another *H* antigen gene, *H47* (unpublished data) were found in all the samples confirming the integrity of the RNA samples. Thus, despite the presence of complementary sequences in the B6 genome, expression of the NS1178 gene was not detectable in the spleen cells.

Taken together, these observations demonstrate that NS1178 cDNA defines the *H28* locus, which is a polymorphic, differentially expressed locus in the BALB and B6 strains. This locus encodes a novel 50 kDa protein with no known function other than serving as a precursor for a naturally processed peptide presented by the K^b MHC molecule.

ILENFPRL Is the Antigenic Octapeptide within NS1178 Presented by the K^b MHC Molecule

Next, we identified the antigenic peptide encoded within the NS1178 cDNA that was presented by the K^b MHC and recognized by the BCZ104.2 T cells. First, using the xxxx(F,Y)xx(I,L,V,M) consensus sequence of K^b binding peptides (Falk et al., 1991), we identified four peptides, K^b1 through K^b4, that matched this sequence (Figures 3A and 3B). Three deletion constructs lacking sequences 3′ to the internal HindIII (ΔHind), XbaI (ΔXba), and BamHI (ΔBam) were prepared and tested for their ability to generate the BCZ104.2-stimulating ligand in recipient cells in the presence of the cotransfected K^b cDNA (Figure 3C, left panel). The response of the BCZ104.2 T cells to the ΔHind and ΔXba but not to ΔBam constructs suggested that the antigenic peptide was encoded in the region flanked by the XbaI and BamHI sites that contained the K^b2 candidate epitope. Indeed, a minigene construct (M-IFL8) encoding only the K^b2 sequence (Figure 3B) with an ATG codon for efficient translation initiation strongly stimulated BCZ104.2 T cells. Most importantly, none of the recipient cells coexpressing the D^b MHC with any of these DNA constructs generated the T cell–stimulating ligand (Figure 3C, right panel). Thus, the K^b MHC as well as the antigen gene were strictly required for BCZ104.2 T cell stimulation.

Conclusive proof that the K^b2 octapeptide was the antigenic peptide was obtained by testing the synthetic ILENFPRL (IFL8) peptide in an exogenous assay (Figure 3D). The synthetic IFL8 peptide stimulated the BCZ104.2 T cells at picomolar concentrations but failed to stimulate the H60-specific BCZ103.1 T cell even at a 100,000-fold higher concentration. As a reciprocal specificity

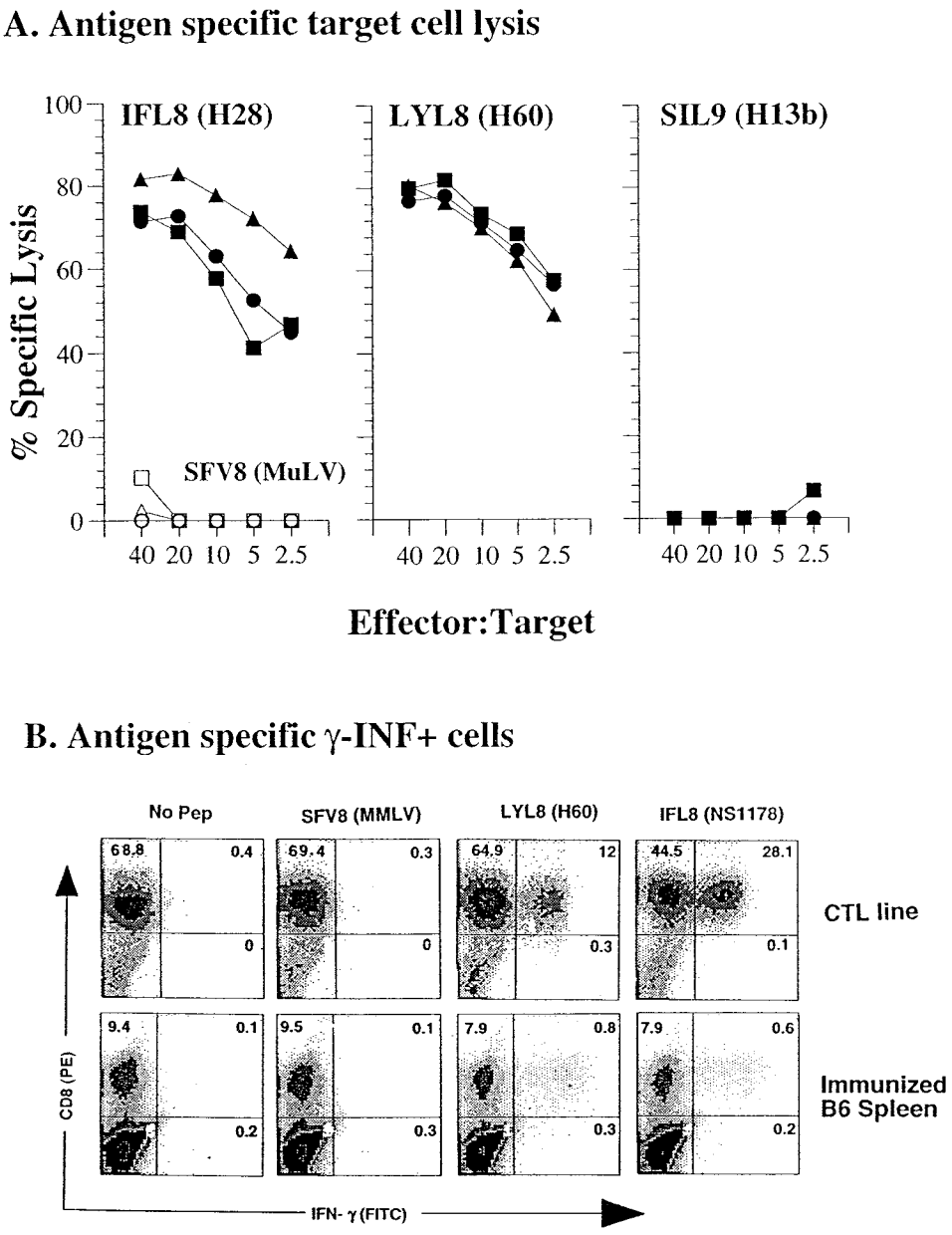

Figure 4. The H28 and H60 Specificities Dominate the B6 Anti-BALB.B CD8 T Cell Response

(A) Antigen-specific target cell lysis by three independently derived B6 anti-BALB.B CTL lines. RMA/S cells were used as targets in the presence of 100 nM concentration of the indicated H antigen peptides. The K^b binding SFV8 peptide, derived from the MuLV gene, was used as a negative control.

(B) Flow cytometric analysis of $CD8^+$ cells that secrete interferon-γ during a 5 hr incubation with the indicated peptides. The results in the top panels were obtained with a bulk B6 anti-BALB.B CTL line and those in the bottom panels with ex vivo spleen cells from B6 mice immunized thrice with BALB.B spleen cells. The numbers indicate the percentage of cells in that quadrant.

control, the BCZ103.1 but not the BCZ104.2 T cells responded to picomolar concentrations of the H60 encoded LYL8 peptide. Addition of a single phenylalanine at the N terminus of the IFL8 peptide (FIL9) caused a greater than 10,000-fold loss of activity. We conclude that IFL8 was the optimal antigenic peptide within the NS1178 cDNA that was presented by the K^b MHC to the BCZ104.2 T cells.

To further confirm that the H28 locus was defined by the expression of the antigenic IFL8 peptide, we immunized B6 mice with spleen cells from the congenic B6.C$^-$*H28* strain. The CTLs lysed T2-K^b target cells incubated with 10 nM IFL8 peptide (specific lysis = 43.4 ± 1.5%) compared to lysis of the same target cells incubated with the irrelevant K^b binding VSV peptide (specific lysis = 0.6 ± 0.1%). Thus, the NS1178 and the IFL8 peptide defined the molecular basis for the polymorphic *H28* locus in the BALB background.

The Anti-H28 and Anti-H60 Specificities Dominate the B6 Anti-BALB.B CTL Response

The H28 and H60 antigens were identified using individual CD8$^+$ T cell clones in the B6 anti-BALB.B CTL population. To determine if these two specificities were frequent, we analyzed polyclonal B6 anti-BALB.B CTL. First, three independently derived bulk CTL lines from female B6 mice immunized with male BALB.B spleen cells were tested for their lytic activity against H-2^b target cells incubated with the IFL8 (H28), LYL8 (H60), or the SIL9 (H13^b) peptides in a JAM assay (Matzinger, 1991) (Figure 4A). A high level of IFL8- and LYL8-specific lytic activity was found in all three CTL lines that had been restimulated six to eight times in vitro. The cytolytic activity was specific because it was not observed toward target cells in the presence of SFV8, an irrelevant K^b binding peptide. Significantly, lytic activity was not detected toward target cells incubated with the SIL9 peptide, which represents the *H13* histocompatibility polymorphism between the B6 and BALB.B strains nor to the Uty derived WI9 peptide (data not shown) (Greenfield et al., 1996; Mendoza et al., 1997). Thus, anti-H28 and anti-H60 CTL were reproducibly elicited in the B6 anti-BALB.B response and dominated the response to other known H antigens.

The frequency of H28- and H60-specific clones among the B6 anti-BALB.B CTLs was determined by analysis of T cell hybridomas generated by fusing each of the three CTL lines with the BWZ36/CD8α fusion partner. Analysis of individual hybridomas showed 22% (24) and 37% (40) of 106 anti-BALB.B hybrids tested were specific for the H28 and H60 derived LYL8 and IFL8 peptides, respectively (data not shown). To ensure that this estimate reflected the actual frequency of T cells specific for these antigens, we also determined the frequency of CD8$^+$ T cells that produced interferon-γ after antigen stimulation of the bulk CTL line. Again, 16% and 39% of all CD8$^+$ T cells produced interferon-γ when they were stimulated with the LYL8 and IFL8 peptides relative to negligible responses to no peptide or to an irrelevant K^b binding SFV8 peptide (Figure 4B). Further evidence that these T cells arose during the immune response in the animal rather than being enriched during in vitro culture was obtained by measuring the frequency of IFNγ^+ CD8$^+$ T cells ex vivo. Remarkably, IFNγ^+ anti-LYL8 and anti-IFL8 CD8$^+$ T cells were readily detected in the immunized spleens at a frequency of 7%–10% of the total CD8$^+$ T cells. We conclude that the anti-H28 and anti-H60 T cell clones dominate the host B6 CTL response to the donor BALB.B cells.

H Antigen Expression Can Be Constitutive or Induced

To assess the potential factors that could contribute to the immunodominance of the H28 and H60 antigens, we first analyzed their expression in different cell types. In initial experiments, we noticed that both a macrophage (RAW309Cr.1) and a pre-B (C3) cell line stimulated the H60-specific T cell hybridoma clones but failed to stimulate the H28-specific T cell hybrid. However, antigen presentation activity was strongly induced in the macrophage line, in contrast to the pre-B cell line, upon incubation with a panel of inflammatory cytokines (Figure 5). Expression of H28-specific antigen presentation was strongly induced with all the inteferons (α, β, and γ) tested, but not by TNFα. Furthermore, the H3^a antigen was constitutively expressed in both cell lines, but H-Y antigen expression was observed only in the pre-B cell line and not in the RAW cells even in the presence of the cytokines. This result suggested that the expression of H antigens was not ubiquitous and constitutive but was cell type specific and inducible.

We then examined the expression of these four H antigens in different tissues isolated from male BALB.B donor cells. The H3^a autosomal antigen is present in both B6 and BALB.B strains (Zuberi et al., 1998), but the H-Y antigen is expressed only in male cells (Greenfield et al., 1996). The naturally processed peptides of H3^a (ACL9) and HY (WI9) are presented by the D^b MHC I molecule present in both B6 and BALB.B strains. Accordingly, untreated or cytokine-treated female B6 spleen cells stimulated only the H3^a-specific, but not the H28-, H60-, or the HY-specific T cell hybrids. In the presence of the appropriate peptide, all the T hybrids responded strongly to the B6 spleen cells. In contrast, the BALB.B spleen cells failed to stimulate the H28-specific T cells but stimulated the H60-, H3^a-, and HY-specific T cells without any cytokine treatment. Again, when the spleen cells were pretreated with IFNα, β, or γ, but not TNFα, the H28-specific T cells responded strongly. The bone marrow cells showed a similar reactivity pattern with some quantitative differences in the basal and cytokine-induced responses. We conclude that unlike the other constitutively expressed H antigens, expression of the H28 antigen was barely detectable in untreated cells but was strongly induced by certain inflammatory cytokines.

Transcriptional Regulation, Abundance, and MHC Binding Affinity of Naturally Processed H60 and H28 Antigenic Peptides

To assess if the cytokine-induced upregulation of H28-specific T cell activation by BALB.B-derived cells was due to transcriptional activation of the *H28* gene, we analyzed the RNA from the RAW and bone marrow cells after treatment with different cytokines. Northern blot analysis with the NS1178 (H28) probe showed that in both the RAW as well as in bone marrow cells, H28 transcription was barely detectable in resting cells or those treated with TNFα (Figure 6A). In contrast, H28 transcription was strongly induced by all interferons. To determine whether the upregulation of H28 transcription and of APC function led to an increase in the generation of naturally processed peptides, we examined the naturally processed H28 and H60 peptides in HPLC fractionated cell extracts of IFNγ-induced RAW macrophages. A single activity peak was found in the HPLC fractions tested with the H60- and H28-specific BCZ103.1 and BCZ104.2 T cells (Figure 6B). These activity peaks coeluted with the synthetic H60 and H28 derived LYL8 and IFL8 peptides, respectively, indicating that the naturally processed peptides were identical to their synthetic counterparts and that no qualitative changes had occurred in antigen processing. Quantitative estimates of the H60 and H28 peptides in the untreated and interferon-γ treated RAW cell extracts showed that the amount of H60 peptide remained unchanged at $\sim$6–15 copies/cell, but the amount of H28 peptide increased from undetectable to $\sim$200 copies/cell. We conclude that cytokine treatment of macrophages specifically upregulated *H28* gene transcription and as a consequence expression of the IFL8/K^b complex that directly accounted for the augmentation in the T cell response.

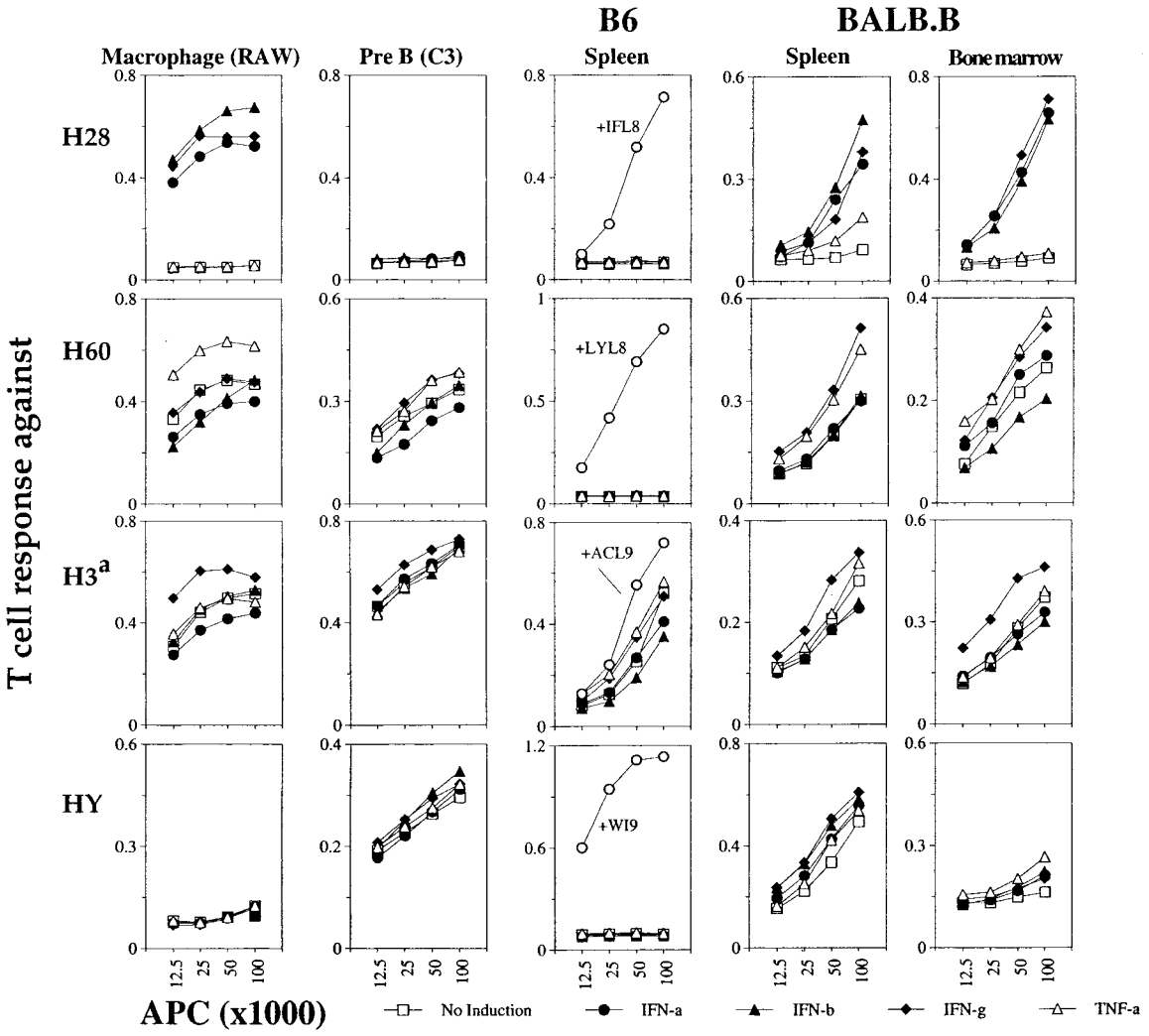

Figure 5. Constitutive and Inducible Expression of H Antigens in Cell Lines and Lymphoid Cells

Expression of H antigens was measured by the ability of different cells to stimulate T cells specific for the H28, H60, $H3^a$, and HY antigens. RAW and C3 are transformed cell lines of the macrophage and B cell lineage. Spleen or bone marrow cells were from either B6 or BALB.B mice. The cells were incubated overnight in either medium alone or with the indicated cytokines, washed, and cocultured with lacZ inducible T cell hybrids specific for each H antigen. As positive controls, the specific H antigen peptide was added to the B6 cells. The lacZ responses were measured as in legend to Figure 1.

In addition to the increase in the overall amount of the naturally processed H28 peptide, it was also possible that the H antigen peptides were bound to MHC molecules with an unusually high affinity. A competitive binding assay was used to compare the binding affinity of H28 and H60 peptides, with other known K^b binding peptides. The specific binding of the mab 25D1.16 to its ligand, the ovalbumin derived SL8 peptide/K^b complex, can be detected by flow cytometry on the surface of RMA/S cells (Figure 6C) (Porgador et al., 1997). The dose-dependent decrease in the mean fluorescence intensity caused by the other peptides is a relative measure of how well these peptides bind K^b MHC. The H28 and H60 derived LYL8 and IFL8 peptides specifically inhibited the binding of SL8 to K^b MHC. The inhibition curves were, however, not significantly different from those obtained with two other peptides of self (SFL8) or viral (SFV8) origin. We conclude that the MHC binding affinity of H28 as well as H60 antigens was comparable to that of other MHC binding peptides.

H Antigens Are Differentially Expressed in Professional APC

To identify the cells that express H antigens, we fractionated the T, B, macrophages, or dendritic cells by flow cytometry using cell type-specific surface markers. The fractionated cells were then used as APCs for stimulating H28-, H60-, $H3^a$ -, or HY-specific T cells as such or after overnight incubation with interferons or TNFα (Figure 7). Fractionated B or T cells were generally poor in their ability to stimulate any of the T cells even after treatment with the cytokines. In contrast, untreated macrophages and dendritic cells were excellent stimulators of H60- and $H3^a$-specific T cells. Expression of the HY/D^b ligand was detected primarily in macrophages

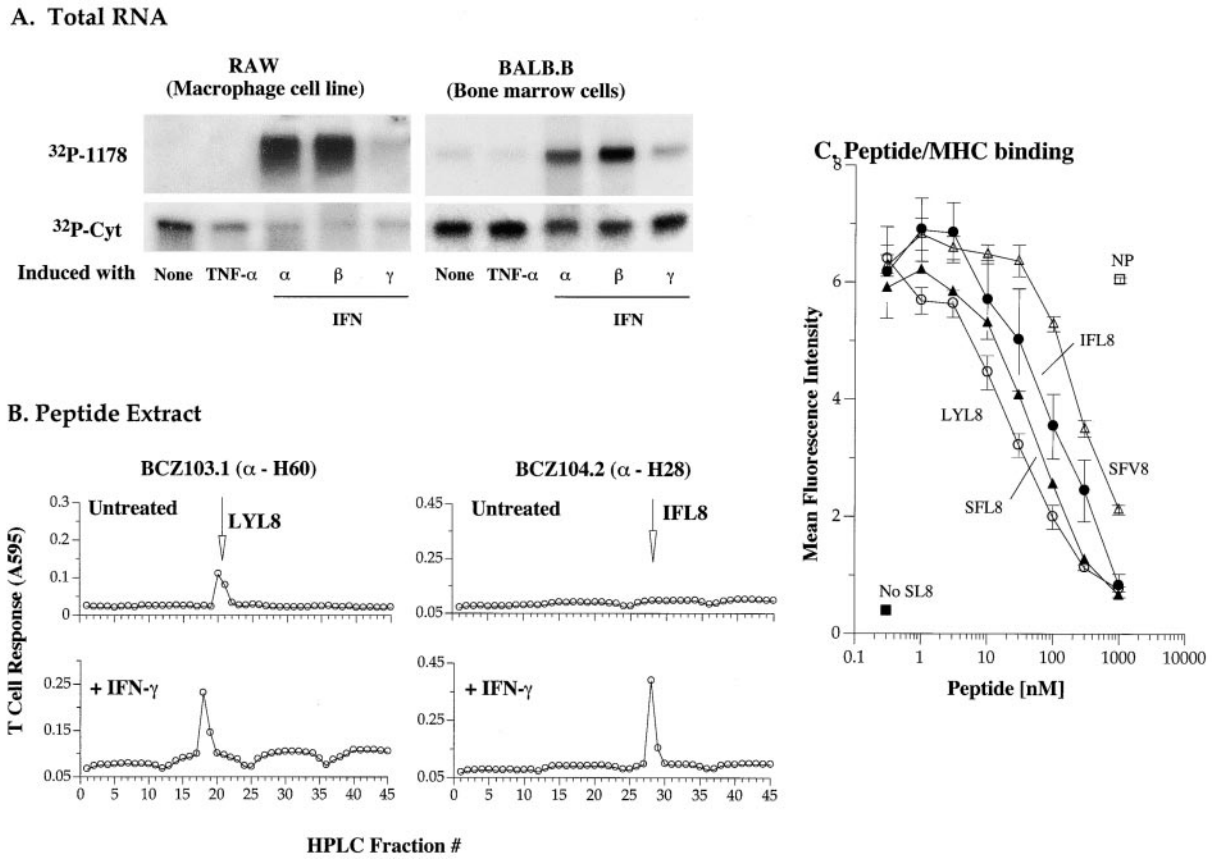

Figure 6. Inflammatory Cytokines Regulate Transcription of the H28 Locus and Increase Expression of Its Naturally Processed IFL8 Peptide

(A) Northern blot analysis of RAW or BALB.B bone marrow cells with the H28 probe. Total RNA was extracted from cells that had been cultured overnight with 100 U/ml of the indicated cytokines. The same blot was also analyzed with a probe for the housekeeping cytochrome c gene as a loading control.

(B) Naturally processed peptide extracts from uninduced and interferon-γ-induced RAW cell line were fractionated by reverse phase HPLC. The fractions were tested for presence of antigenic activity using H60 (BCZ103.1)- or H28 (BCZ104.2)-specific T cells. The peak elution times for the synthetic LYL8 (H60) and IFL8 (H28) run under identical conditions are shown by a vertical arrow.

(C) The relative K^b binding affinity of peptides was assessed using a flow cytometric assay based upon the ability of peptides to compete with the ovalbumin derived SL8 peptide for binding to K^b. Cell surface expression of the SL8/K^b was specifically detected with the 25D1.16 mAb. The peptides were IFL8, ILENFPRL; SFV8, SSWDFITV; LYL8, LTFNYRNL; SFL8, SVVEFTSL. The D^b binding NP peptide, ASNENMETM, was used as a negative control. The lacZ responses were measured as in legend to Figure 1.

and was far lower in the dendritic cells. Most significantly, expression of H28 dramatically and specifically increased in dendritic cells and macrophages after treatment with the interferons but not with TNFα. We conclude that only a subset of H antigens are efficiently expressed in professional APCs.

Discussion

The molecular identity of minor histocompatibility loci and why they cause chronic graft rejection in MHC matched individuals has remained an enduring enigma. We show here that the murine autosomal *H28* locus encodes a previously unknown protein that is the source of the naturally processed IFL8 octapeptide presented by the K^b MHC molecule. The nature of genetic polymorphism at the *H28* locus, and comparison with other *H* loci, provide insights into the sources as well as the mechanisms of immunogenicity and immunodominance of H antigens that are targeted by CTLs.

Molecular Definition of *H* Loci

With tens of thousands of proteins, mammalian cells are the most complex entity in the antigenic universe faced by the immune system. The full brunt of this complexity is realized when host T cells respond with a very high frequency to a multitude of different peptide/donor MHC complexes on allogeneic cells (Lindahl and Wilson, 1977; Rotzschke et al., 1991). In contrast, when the MHC molecules are matched the host T cells respond only to non-MHC antigens–those defined by the *H* loci. There appears to be a large discrepancy between the number of potential *H* loci and those that have been experimentally observed. Currently, more than 4000 polymorphic differences are known to exist between the B6 and BALB genetic backgrounds as defined by PCR or RFLP analysis (http://www.informatics.jax.org/). Although many of these genetic polymorphisms may not be reflected in amino acid substitutions in protein sequences, this number is expected to increase exponentially as genome sequences become available. In contrast, experimental estimates by Bailey and colleagues showed that a mini-

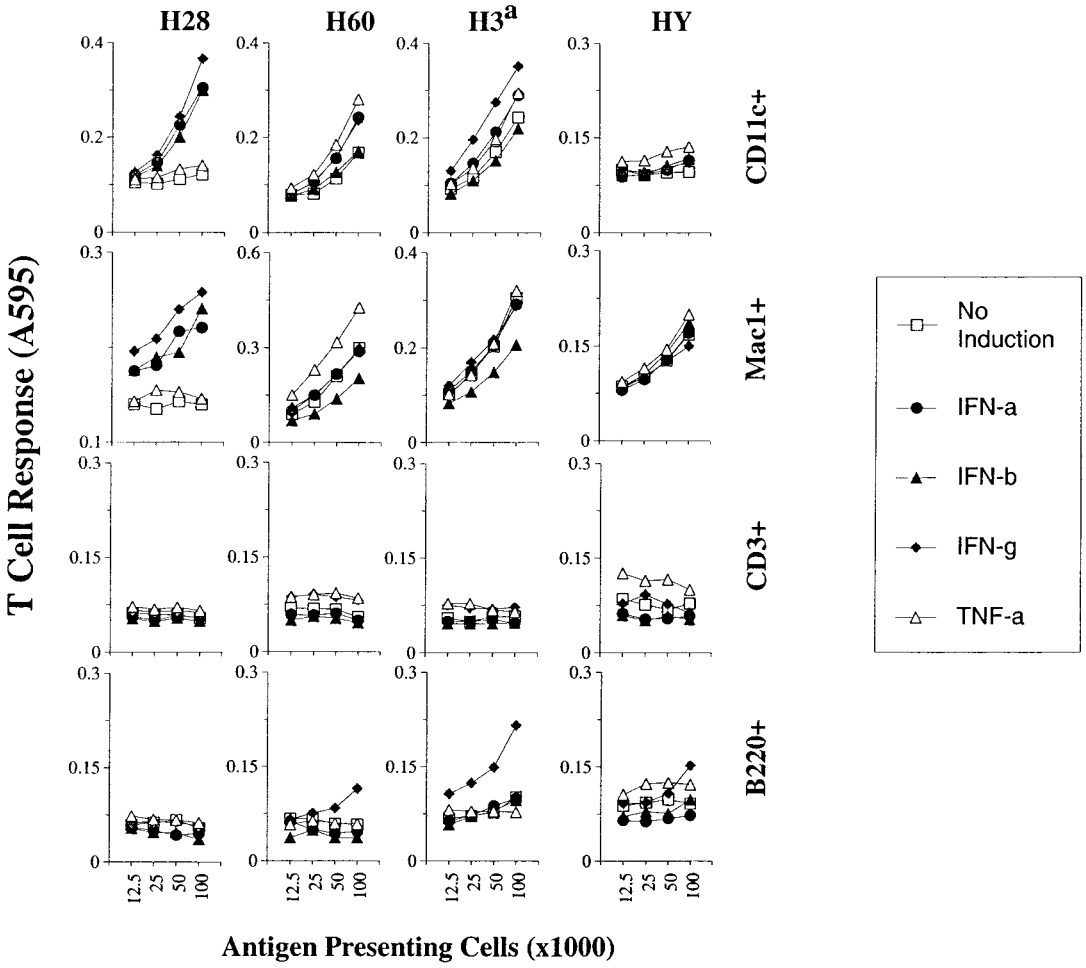

Figure 7. Expression of H Antigens Is Cell Type Specific and Induced by Inflammatory Cytokines

BALB.B cells were sorted according to the expression of surface markers. The cells were incubated overnight in media containing 100 U/ml of the indicated cytokines, washed, and then tested for their ability to stimulate T cell hybrids specific for H28, H60, $H3^a$, or HY antigens. The lacZ response was measured as in legend to Figure 1.

mum of 29 *H* loci can cause rejection of skin grafts between the B6 and BALB.B strains (Bailey and Mobraaten, 1969). However, analysis using CTLs showed that as few as five distinct specificities were dominant in the CTL response to immunization with spleen cells (Wolpert et al., 1995; Nevala and Wettstein, 1996). The *H* loci that encode these dominant H epitopes are thus the key players that stand out among the large number of potential H antigen disparities.

The *H* loci represent natural polymorphisms that are detected by CTL as novel peptide/MHC complexes, but they could have other functional consequences as well. Indeed, it is remarkable that the H60 protein, described here as a source of the immunodominant LYL8/K^b complex, was also recently identified as the ligand for the NKG2D receptor expressed by a subset of murine NK and $CD8^+$ T cells (Cerwenka et al., 2000; Diefenbach et al., 2000). What role the interactions between the H60 ligand and the NKG2D receptor play in the anti-H60 CTL response is not known but could add another dimension to potential mechanisms of immunodominance. For example, the $NKG2D^+$ CD8 T cells could engage not only the LYL8/K^b ligand via their TCR/CD8 receptors but also the cell surface H60 protein itself. The normal cellular functions and the consequences of the polymorphisms in H28 and most other H proteins still remain to be discovered.

All the *H* loci identified so far represent the structural antigen genes that are the precursors for the naturally processed antigenic peptides presented by MHC molecules. Nevertheless, the existence of transcriptionally regulated *H* loci raises the possibility that some *H* loci may encode transcription factors that regulate the expression of structural H antigens in trans. Note also that the existence of transcriptionally regulated *H* loci described here makes it impossible to match tissue donors with the host by a simple test for DNA polymorphisms alone.

Mechanisms of Immunogenicity and Immunodominance of H Antigens

The high fraction of B6 anti-BALB.B CTLs that were specific for the H60 and H28 derived LYL8 and IFL8 peptides was striking. Not only did LYL8- and IFL8-specific CTL account for >50% of all $CD8^+$ T cells in

independently derived CTL lines, but they were also readily detected in ex vivo spleen cells. This analysis of the frequency of antigen-specific CTL specific for transplantation antigens shows that as in responses to viruses or bacteria, certain specificities prevail (Busch et al., 1998; Murali-Krishna et al., 1998). Thus, despite the existence of CTLs specific for the *H13*^{b} and *Uty* derived SIL9 and WI9 peptides, these cells were not detected in the B6 anti-BALB.B response. Therefore, CTL responses in the host do not include all possible foreign epitopes in the donor but focus primarily on epitopes derived from *H28* and *H60* genes.

Two different mechanisms have emerged to explain why donor H antigens are immunogenic in the host and why the CTL responses are uni- or bidirectional. The first mechanism was revealed by the identification of the antigenic peptide derived from the mitochondrial protein ND1 (Loveland et al., 1990). Polymorphisms among the natural ND1 alleles results in presentation of peptides with a single amino acid substitution by the nonclassical H2-M3 MHC molecule. Because both the donor and host present self-ND1 peptides, reciprocal immunizations elicit CTL responses specific for the donor ND1/H2-M3 complex–the CTL responses are bidirectional. A similar pattern of biallelic expression was also observed for the autosomal *H13* and *H3*^{a} genes (Mendoza et al., 1997; Zuberi et al., 1998). In contrast, the female anti-male CTL responses to the HY antigens are unidirectional because only the Y chromosome encoded *Uty* and *Smcy* genes yield unique antigenic peptide/MHC complexes (Scott et al., 1995; Greenfield et al., 1996). However, these genes do have X chromosome homologs (*Smcx* and *Utx*) expressed in female cells, but they do not yield antigenic peptide/MHC complexes because of substitutions that abrogate MHC binding. The autosomal *H28* and *H60* genes are the most extreme as they are the first examples of donor *H* loci that are effectively null alleles in the host. The reasons for this differential transcriptional regulation in the BALB and B6 backgrounds remain to be determined but provide the explanation for why unique H28 and H60 derived IFL8 and LYL8 peptides are displayed by the K^b MHC in the donor BALB.B cells.

One of the explanations proposed for immunodominance is the binding characteristics of the antigenic peptide to MHC protein (Nevala and Wettstein, 1996; Sijts and Pamer, 1997; Chen et al., 2000). We failed to detect a significant difference between the binding affinity of the IFL8 and LYL8 peptides for the K^b MHC relative to other known peptides. Furthermore, neither of these two naturally processed peptides was expressed at unusually high levels. In fact, the IFL8 peptide was barely detected in functional or biochemical assays using resting APC. Expression of IFL8 was, however, strongly induced after the cells were cultured with α, β, or γ interferons. The increase in IFL8/K^b-specific T cell activation could be accounted for by upregulation of *H28* gene transcription and was reflected in the enhanced abundance of the naturally processed peptide in cell extracts. Thus, neither high MHC binding affinity nor the natural abundance is adequate explanations for the immunodominance of the H28 and H60 epitopes.

Could expression by professional APCs explain immunodominance? Interestingly, we discovered that both H60 and H28 epitopes were expressed in bone marrow–derived dendritic cells either constitutively or after interferon stimulation. By contrast, the subdominant HY antigen expression was barely detected in the DC and it did not increase after interferon stimulation. Resting T or B cells were uniformly low in their ability to present either H28, H60, H3^a, or HY peptides to appropriate T cells. These findings demonstrate that H antigens are selectively expressed in certain cell types and provide a potentially powerful mechanism for immunodominance. Because dendritic cells are believed to be professional APC that initiate naïve CD8 T cell responses in vivo, we postulate that H antigens that are directly expressed in donor dendritic cells have an advantage over those H antigens that are not. An antigen such as *H28* also belongs to the former category because its expression can be induced by inflammatory cytokines that are inevitably produced during experimental procedures of surgery or injection. CTL responses to H antigens that are poorly expressed by donor DC would lead to less efficient immunity and/or require that the host DCs acquire these antigens via the "cross-priming" mechanism. While this hypothesis provides a molecular explanation for the high immunogenicity of "passenger leukocytes," now known to be DCs, it also raises the key question of the relative efficiency of direct versus cross-priming mechanisms in generating CTL responses to H antigens (Bevan, 1976). The knowledge of dominant and subdominant H antigen genes, proteins, and their naturally processed peptides now makes it possible to address these hypotheses.

Experimental Procedures

Animals, Immunizations, and Cell Lines

Inbred mouse strains C57BL/6, 129/J, BALB.B, and B6.C-H28^c were obtained from or bred at The Jackson Laboratory (Bar Harbor, ME) or at the animal care facility at the University of California, Berkeley, CA. Immunizations, generation, and maintenance of cell lines and T cell hybrids has been described (Karttunen et al., 1992; Sanderson and Shastri, 1994; Malarkannan et al., 1998). The (BALB/cxBALB.B)F1 derived RAW309Cr.1 cells were from ATCC.

T Cell Activation Assays

T cell responses specific for peptide/MHC were measured by the production of β-galactosidase (LacZ) activity in the T cell hybrids (Sanderson and Shastri, 1994). T cell hybrids ($3–10 \times 10^4$) were cocultured overnight with APC ($2–5 \times 10^4$) either expressing the antigen endogenously, transfected with antigen cDNA or with exogenous peptides in 96-well plates. The peptide/MHC-induced T cell response was assayed as lacZ activity using the substrate chlorophenol red β-D-galactopyrannoside (CPRG). The conversion of CPRG to chlorophenol red was measured at 595 and 655 nm as a reference wavelength with a 96-well microplate reader (BioRad, Richmond, CA). Cytolytic activity was detected by the JAM assay using ^{3}H-thymidine labeled RMA/S cells in the presence of 100 nM concentration of the indicated peptides (Matzinger, 1991).

cDNA Library, Expression Screens, DNA Constructs, and Peptides

Construction of unidirectional cDNA libraries and screens for isolating T cell–stimulating antigen clones have been described (Karttunen et al., 1992; Shastri, 1996; Malarkannan et al., 1998). Deletion constructs were prepared in the pcDNAI vector using internal restriction sites. The minigene constructs encoding K^{b}1, HSISYRNM; K^{b}2, MILENFPRL; K^{b}3, LGHGFLPV; and K^{b}4, AYKPYKDL and the H60 antigenic peptide, MLTFNYRNL, were prepared using complementary oligonucleotides corresponding to the indicated sequences. The peptides, ILENFPRL (IFL8), LTFNYRNL (LYL8), SSWDFITV (SFV8), SIINFEKL (SL8), ASNENMETM (NP), and SSVIGVWYL (SIL9) were synthesized, purified by HPLC, and confirmed by mass spectrometry.

In Vitro Translation and Genetic Mapping

In vitro translation reactions were carried out with the indicated cDNA clones and the TnT kit from Promega according to manufacturer's instructions. Southern and Northern blots analysis was carried out according to established procedures using the indicated cDNA fragments as the ^{32}P-labeled probes (Ausubel et al., 1994).

MHC Binding and HPLC Analysis of Naturally Processed Peptides

The synthetic IFL8, LYL8, SFV8, and SEL8 peptides were compared for their relative K^b binding affinity by flow cytometry using RMA/S cells and the recently described antibody, 25D1.16, that recognizes the SL8/K^b complex (Porgador et al., 1997; Malarkannan et al., 1998). Naturally processed peptides were analyzed in cell extracts by reverse phase HPLC as described earlier (Malarkannan et al., 1998, 1999).

Cell Fractionation

$CD3^+$ and $B220^+$ cells were sorted from cells pooled from spleen and lymph nodes, $CD11c^+$ cells from bone marrow cells cultured for 7 days in media containing conditioned L-cell supernatant. $Mac1^+$ cells were from peritoneal exudate cells. Anti-CD3 antibody, anti-B220, anti-CD11c, and anti-MAC1 antibodies were obtained from Pharmingen. Prior to staining with specific antibodies, cells were incubated with rabbit serum to prevent nonspecific antibody binding. Stained cells were sorted by FACS and incubated overnight with the 100 U/ml of the indicated cytokines (Pharmingen) and used as APC.

Enumeration of Antigen-Specific CTL by Intracellular IFNγ Staining

CTL lines or spleen cells from the immunized animals were plated out in 96-well U-bottom plates ($\sim 1 \times 10^6$/well), and incubated for 5 hr at 37° with IL-2 (10 U/ml), Golgi Plug (Pharmingen, San Diego, CA, 1 ul/ml) to block cytokine secretion. The cells were cultured in medium alone or with 200 nM of synthetic peptides for 5 hr. The cells were then stained first with PE-conjugated, anti-CD8 antibody, fixed, permeabilized, and then stained with FITC-conjugated, anti-IFNγ antibody. All reagents were from the Intracellular IFNγ staining kit from Pharmingen and used according to instructions. Stained cells were analyzed by flow cytometry to enumerate the fraction of $CD8^+$ cells that also contained intracellular interferon-γ.

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